


FORM PTO-1390 (REV. 12-29-99)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER YAM 2 0010	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				U.S. APPLICATION NO. (if known, see 37 CFR 1.5) <b>09/914546</b>	
INTERNATIONAL APPLICATION NO. PCT/JP99/03920		INTERNATIONAL FILING DATE 22 July 1999		PRIORITY DATE CLAIMED None claimed	
TITLE OF INVENTION ULTRA-FAST TRANSFORMATION TECHNIQUE FOR MONOCOTYLEDONS					
APPLICANT(S) FOR DO/EO/US TANAKA et al.					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
<ol style="list-style-type: none"><li>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</li><li>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</li><li>3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).</li><li>4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</li><li>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))<ol style="list-style-type: none"><li>a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).</li><li>b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau.</li><li>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</li></ol></li><li>6. <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).</li><li>7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))<ol style="list-style-type: none"><li>a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</li><li>b. <input type="checkbox"/> have been transmitted by the International Bureau.</li><li>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</li><li>d. <input type="checkbox"/> have not been made and will not be made.</li></ol></li><li>8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</li><li>9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). (UNEXECUTED)</li><li>10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</li></ol>					
Items 11. to 16. below concern document(s) or information included:					
<ol style="list-style-type: none"><li>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</li><li>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</li><li>13. <input type="checkbox"/> A <b>FIRST</b> preliminary amendment. <input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment.</li><li>14. <input type="checkbox"/> A substitute specification.</li><li>15. <input type="checkbox"/> A change of power of attorney and/or address letter.</li><li>16. <input type="checkbox"/> Other items or information:</li></ol>					
<p>"Express Mail" Mailing Label Number <u>EL 852673355 US</u> Date of Deposit <u>8/28/2001</u> I hereby certify that this paper of fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.</p> <p><u>LAURIE A. BOYLAN</u> (TYPED OR PRINTED NAME OF SENDER) <u>Laurie A. Boylan</u> (SIGNATURE)</p>					

US.

US APPLICATION NO. (if known) <b>09/914546</b>		INTERNATIONAL APPLICATION NO. <b>PCT/JP99/03920</b>		ATTORNEY'S DOCKET NUMBER <b>YAM 2 0010</b>	
17. <input checked="" type="checkbox"/> The following fees are submitted: <b>BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :</b> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... \$970.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO. .... \$840.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$690.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... \$670.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) ..... \$96.00  <b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>				<b>CALCULATIONS</b> PTO USE ONLY	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	5 - 20 =	0	X \$18.00	\$	
Independent claims	1 - 3 =	0	X \$78.00	\$	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$260.00	\$	
<b>TOTAL OF ABOVE CALCULATIONS =</b>				\$	
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				\$	
<b>SUBTOTAL =</b>				\$	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$	
<b>TOTAL NATIONAL FEE =</b>				\$ 860.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property				\$	
<b>TOTAL FEES ENCLOSED =</b>				\$ 860.00	
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b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.					
c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>06-0308</u> . A duplicate copy of this sheet is enclosed.					
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.					
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### **Application Information**

Title Line One:: ULTRA-FAST TRANSFORMATION TECHNIQUE  
Title Line Two:: FOR MONOCOTYLEDONS  
Total Drawing Sheets:: 3  
Formal Drawings?: Yes  
Application Type:: Utility

### **Representative Information**

Registration Number:: 33,000

### **Continuity Information**

This application is a:: U.S. National Phase of  
> Application One:: PCT/JP99/03920  
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## DESCRIPTION

### ULTRA-FAST TRANSFORMATION TECHNIQUE FOR MONOCOTYLEDONS

#### 5 TECHNICAL FIELD

The present invention relates to an Agrobacterium-mediated transformation method for monocotyledons.

#### BACKGROUND ART

10 One means for improving a plant is a "transformation technique", in which a desired recombinant gene for modifying a character is introduced into a plant. Efficient and quick transformation techniques are extremely important for the molecular  
15 breeding of useful plants, in particular grain crops, which are important staple foods.

A majority of grain crops (e.g., rice, wheat, barley, and corn) are classified as monocotyledons.  
20 Various transformation techniques for transforming monocotyledons have hitherto been developed. Transformation techniques are generally classified into direct transformation techniques and indirect transformation techniques.

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Examples of direct transformation techniques include electroporation techniques (see Shimamoto K. et al., Nature, 338:274-276, 1989; and Rhodes C.A. et al., Science, 240:204-207, 1989), particle gun techniques (see Christou P. et al., Bio/Technology 9:957-962, 1991) and polyethylene glycol (PEG) technique (see Datta, S.K. et al., Bio/Technology, 8:736-740, 1990). Electroporation techniques and particle gun techniques have been generally used as methods for transforming monocotyledons which can achieve a relatively efficient gene introduction.

An example of an indirect transformation technique is an Agrobacterium-mediated transformation technique (hereinafter, this may also be referred to as an "Agrobacterium transformation technique"). Agrobacteria are a kind of plant pathogenic bacteria. Agrobacteria are characterized in that, when a plant is infected therewith, a T-DNA region which is present on the plasmids that Agrobacteria have (e.g., Ti plasmid or Ri plasmid) is incorporated into the plant. The Agrobacterium transformation technique utilizes the incorporation of the T-DNA region into plants as a means

for introducing genes into plants. In short, a plant is infected with an Agrobacterium which contains a desired recombinant gene. After infection, a desired recombinant gene is transferred from the Agrobacterium into plant cells so as to be incorporated into the plant genome.

The Agrobacterium transformation technique is sufficiently established so far as dicotyledons are concerned. A large number of stable transformed plants have already been created which express desired recombinant genes.

On the contrary, it has conventionally been recognized as difficult to apply the Agrobacterium transformation technique to monocotyledons. For example, Portrykus et al. (BIO/TECHNOLOGY, 535-542, 1990) report that monocotyledons cannot be infected with Agrobacteria. On the other hand, a great deal of attempts have been made to transform monocotyledons by using Agrobacteria, which have shed light to the possibility of applying the Agrobacterium transformation technique to monocotyledons.

For example, Raineri et al. took the blastodisk



of rice, scarred it, and placed it on a medium which induces regeneration; a few days later, the blastodisk portion was infected with an Agrobacterium. As a result, although normally regenerated plant bodies were not obtained, 5 calluses having a foreign gene introduced therein were successfully induced (see Raineri, D.M. et al., Bio/Technology, 8:33-38, 1990).

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The pamphlet of International Publication 10 No. W094/00977 discloses an Agrobacterium transformation technique for rice and corn. According to this method, it is necessary to employ cultured tissue (e.g., calluses), which is in the process of regeneration or which have completed regeneration, as a plant sample to be 15 transformed by an Agrobacterium. Therefore, prior to infection with an Agrobacterium, it takes 3 to 4 weeks to induce regeneration in order to produce regenerated culture tissue from a plant sample to be transformed (e.g., a leaf section).

20

The pamphlet of International Publication No. W095/06722 discloses a method which infects an immature germ of rice or corn with an Agrobacterium. However, it is quite cumbersome to isolate immature germs.

Accordingly, it will contribute to the molecular breeding of useful monocotyledons, including grain crops such as rice, if a quicker and efficient Agrobacterium transformation technique for monocotyledons becomes available.

#### DISCLOSURE OF THE INVENTION

The present invention is intended to solve the aforementioned problems. An objective of the present invention is to improve Agrobacterium transformation techniques for monocotyledons. According to the method of the present invention, it is possible to create a transformed plant far more efficiently and quickly than any conventional Agrobacterium transformation techniques.

The present invention relates to a transformation method for monocotyledons, the method including a step of infecting an intact seed with an Agrobacterium which includes a desired recombinant gene. According to the method of the present invention, a seed is infected in an intact state, and no processing such as regeneration of a plant sample to be transformed is required.

The seed to be infected with an Agrobacterium may be a seed on the fourth day to the fifth day after sowing. At the time of infection, the seed may already have  
5 germinated.

The monocotyledon to be transformed is preferably a plant of the family Gramineae, and more preferably rice (*Oryza sativa* L.).

10

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a photograph showing a state of a living organism, illustrating the state of a rice seed immediately before infection with an Agrobacterium.

15

Figure 2 is a photograph showing a state of a living organism, illustrating a regenerated rice individual obtained by the method according to the present invention, on about the 50th day after sowing.

20

Figure 3(a) is a photograph showing a state of living organisms, illustrating a transformed plant body obtained by a conventional technique on about the 90th day after sowing, in comparison with a transformed plant

body obtained by the method according to the present invention on about the 50th day since sowing.

Figure 3(b) is a photograph showing a state of living organisms, illustrating a transformed plant body obtained by a conventional technique on about the 50th day after sowing, in comparison with a transformed plant body obtained by the method according to the present invention on about the 50th day after sowing.

10

#### BEST MODES FOR CARRYING OUT THE INVENTION

Hereinafter, the present invention will be described in detail.

15

The "plants" to which the method according to the present invention is applied are monocotyledons. Examples of preferable monocotyledons include plants of the family Gramineae (e.g., rice and corn). The most preferable plant to which the method according to the present invention is applicable is rice, and in particular Japonica rice. Unless otherwise indicated, the term "plant" means a plant body and the seeds obtained from a plant body.

20

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(Preparation of a plant expression vector)

In order to introduce a desired recombinant gene into monocotyledons, an appropriate plant expression vector which includes the desired recombinant gene is constructed. Such a plant expression vector may be prepared by recombinant gene technologies well-known to those skilled in the art. Although pBI-type vectors are suitably employed to construct plant expression vectors for use in Agrobacterium transformation techniques, the plant expression vector is not limited thereto.

A "desired recombinant gene" refers to any polynucleotide which is desired to be introduced into a plant. The desired recombinant gene according to the present invention is not limited to those isolated from nature, but may also include synthetic polynucleotides. Synthetic polynucleotides may be obtained by, for example, synthesizing or modifying a gene having a known sequence by techniques well-known to those skilled in the art. The desired recombinant genes according to the present invention include, for example, any polynucleotide which is desired to be expressed in a plant to be transformed that may be endogenous or exogenous to that plant, or, in the case where it is desired to control the expression

of a certain endogenous gene in a plant, a polynucleotide which includes an antisense sequence of a target gene.

In the case where expression in a plant is intended,  
5 a desired recombinant gene may contain its own promoter (i.e., the promoter to which the gene is operably linked in nature) in an operable manner, or, in the case where the gene's own promoter is not contained or where it is desirable that a promoter other than the gene's own  
10 promoter be contained, operably linked to any appropriate promoter. Examples of the promoter to be used include, a constitutive promoter, a promoter which is selectively expressed in a portion of a plant body, and an inducible promoter.

15

In the plant expression vector, various regulatory elements may further be linked in a such manner as to be operable within the host plant cells. The regulatory elements may include, preferably, selection  
20 marker genes, plant promoters, terminators, and enhancers. It is well-known to those skilled in the art that the type of the plant expression vector to be used and the kinds of regulatory elements may vary depending on the purpose of transformation.

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A "selection marker gene" can be used in order to facilitate the selection of transformed plants. Drug resistance genes such as a hygromycin phosphotransferase(HPT) gene for imparting hygromycin resistance, and a neomycin phosphotransferase II(NPTII) gene for imparting kanamycin resistance, may be suitably employed, although not limited thereto.

10 A "plant promoter" means a promoter which is operably linked to a selection marker gene and expressed in a plant. Examples of such promoters include cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthetase promoter, although not limited thereto.

15 A "terminator" is a sequence which is located downstream of a region of a gene which codes for a protein, and which is involved in the termination of transcription when DNA is transcribed to mRNA, as well as the addition of a polyA sequence. Examples of terminators include 20 CaMV35S terminator and the nopaline synthetase terminator (Tnos), although not limited thereto.

An "enhancer" may be employed in order to enhance

the expression efficiency of a target gene. As the enhancer, an enhancer region which includes an upstream sequence within the CaMV35S promoter is suitably used. A plurality of enhancers may be employed for each plant  
5 expression vector.

(Transformation of plants)

The Agrobacterium used for the transformation of monocotyledons may be any bacterium of the genus  
10 Agrobacterium, and preferably Agrobacterium tumefaciens. The Agrobacterium may be transformed by a plant expression vector containing a desired recombinant gene (e.g., by electroporation). By infecting a seed with the transformed Agrobacterium, the desired recombinant gene can  
15 be introduced into the plant. The introduced recombinant gene exists in a form integrated within the genome in the plant. The genome in the plant not only means chromosomes in the nucleus, but also includes genome included in various organelles (e.g., mitochondria, chloroplasts) in  
20 plant cells.

After removing the husks of the seed of a plant which is intended to be transformed, the seed is pre-cultured in an intact state. A seed being "intact" means



that the seed has not been subjected to any artificial manipulations, such as removal of the ovule or scarring of the blastodisk.

5           In the pre-culture, the seeds are sown on a medium (e.g., an N6D medium) containing an appropriate concentration of auxin (e.g., 2,4-D), and may be incubated for typically 4 to 5 days, and preferably 5 days. The pre-culture is completed before the seed tissue enters  
10 into a regeneration process. The temperature during the pre-culture is typically 25°C to 35°C, and preferably 27°C to 32°C. After completing the pre-culture, the seeds are sterilized, and thereafter washed well with water. Next, the seeds may be infected with a transformed Agrobacterium  
15 under aseptic manipulation.

          During infection by the Agrobacterium (co-culture), the seeds are incubated in the dark, typically for 2 to 5 days, and preferably for 3 days. The  
20 temperature at this time is typically 26 °C to 28°C, and preferably 28 °C. Next, in order to eliminate the Agrobacterium in the medium, the seeds are subjected to a treatment with an appropriate bacteria eliminating agent (e.g., carbenicillin). The transformed seeds are

selected on the basis of a selection marker (e.g., drug resistance such as hygromycin resistance).

After the culture under appropriate bacteria-  
5 eliminating conditions and selection conditions, the  
selected transformed seeds may be placed in a regeneration  
medium (e.g., an MS medium) containing appropriate plant  
regulatory substances, and incubated for an appropriate  
period of time. In order to allow a plant body to be  
10 regenerated, the regenerated transformant is placed on  
a rooting medium (e.g., an MS medium containing no plant  
regulatory substances). After the growth of roots is  
confirmed, the transformant may be potted.

15 The desired recombinant gene which has been  
introduced into the plant may have action for intended  
purposes (e.g., expression of a new character of interest,  
or controlling the expression of certain endogenous  
genes) within the plant.

20

It can be confirmed by using well-known techniques  
whether or not a desired recombinant gene has been  
introduced into a plant. This confirmation may be made,  
for example, via Northern Blot analysis. Specifically,

the entire RNA is extracted from a leaf of a regenerated plant, subjected to electrophoresis on agarose in denatured condition, and thereafter blotted on an appropriate membrane. By allowing a labeled RNA probe  
5 which is complementary to a portion of the introduced gene to hybridize with the blots, the mRNA of the gene of interest can be detected. Alternatively, in the case where controlling the expression of an endogenous gene in the plant is desired via the introduction of a desired  
10 recombinant gene, the expression of the target endogenous gene may be tested for example, via the aforementioned Northern Blot analysis. If the expression of the target endogenous gene is significantly suppressed as compared to its expression in a non-transformed control plant, it  
15 is confirmed that the desired recombinant gene has been introduced into the plant and that the desired recombinant gene has acted to control the expression.

Conventional methods usually require a period of  
20 3 to 4 weeks for inducing regeneration prior to Agrobacterium infection. In contrast, the method according to the present invention does not require a step of inducing regeneration, so that the number of days required for creating transformation monocotyledons can

be reduced. Furthermore, according to the method of the present invention, it is also possible to reduce the period which is required for selection by conventional techniques, so that it is possible to reduce the  
5 influences of culture variation.

In a preferable embodiment of the method according to the present invention, the number of days required for creating a transformation monocotyledon is about 50 days,  
10 which is about two-thirds or less of the number of days (about 90 days) required by conventional Agrobacterium transformation methods (see, for example, Example 2 below). Moreover, according to the method of the present invention, a transformation efficiency of 10% to 15% can  
15 be obtained in the case of Nipponbare seeds. A similarly high transformation efficiency can also be achieved with other rice cultivars such as Dontokoi or Kitaake. Therefore, by using the method according to the present invention, it is possible to more efficiently and quickly  
20 create a transformed plant than by conventional transformation techniques.

(Examples)

Hereinafter, the present invention will be

specifically described with respect to examples, which do not limit the present invention. The materials, reagents, and the like which are used in the example are available from commercial sources unless otherwise  
5 specified.

Example 1: transformation of a rice plant using the method according to the present invention

10 After removing the husks of the seeds of Nipponbare, a typical rice cultivar, the seeds in an intact state were sterilized in a 2.5% sodium hypochlorite (NaClO) solution. After being washed well with water, the rice was subjected to the following aseptic  
15 manipulations.

#### 1.1 pre-culture

The seeds were sown on an N6D medium containing 2,4-D (30 g/l of sucrose, 0.3 g/l of casamino acid,  
20 2.8 g/l of proline, 2 mg/l of 2,4-D, 4 g/l of gellite, pH5.8), and incubated at 27°C to 32°C for 5 days. The seeds germinated during this period (Figure 1).

#### 1.2 plant expression vector

As a plant expression vector for transforming an Agrobacterium, a plasmid pIG121Hm, in which the GUS gene including a first intron of the Ricinus catalase gene and a hygromycin resistance gene are linked, was used (Nakamura et al., PLANT BIOTECHNOLOGY II, a special issue of Gendai Kagaku, pp.123-132 (1991)). Agrobacterium EHA101 was transformed with pIG121Hm (Hood et al., J. Bacteriol., 168:1291-1301(1986)). EHA101 is a bacterium in which a vir region of a helper plasmid is derived from the strongly pathogenic Agrobacterium A281.

### 1.3 Agrobacterium infection

In a suspension of the transformed Agrobacterium, the aforementioned pre-cultured seeds were immersed. Thereafter, this was placed on a 2N6-AS medium (30 g/l of sucrose, 10 g/l of glucose, 0.3 g/l of casamino acid, 2 mg/l of 2,4-D, 10 mg/l of acetosyringon, 4 g/l gellite, pH5.2). This was incubated in the dark at 28°C for 3 days to effect co-culturing.

### 1.4 bacterium elimination and selection

After completing the co-culture, an N6D medium containing 500 mg/l of carbenicillin, the Agrobacterium was washed off the seeds. Next, the selection of the

transformed seeds was performed under the following conditions.

First selection: the seeds were placed on an N6D  
5 medium containing 2 mg/l of 2,4-D which was supplemented  
with carbenicillin (500m g/l) and hygromycin (25 mg/l),  
and incubated at 27°C to 32°C for 7 days.

Second selection: the seeds were placed on an N6D  
10 medium containing 2 to 4 mg/l of 2,4-D which was  
supplemented with carbenicillin (500m g/l) and  
hygromycin (25 mg/l), and incubated at 27°C to 32°C for  
7 more days.

15 1.5 regeneration

The selected transformed seeds were allowed to  
regeneration under the following conditions.

First regeneration: the selected seeds were  
20 placed on a redifferentiation medium (an MS medium  
(30 g/l of sucrose, 30 g/l of sorbitol, 2 g/l of casamino  
acid, 2m g/l kinetin, 0.002 mg/l NAA, 4 g/l gellite,  
pH5.8) supplemented with carbenicillin (500 mg/l) and  
hygromycin (25 m g/l)), and incubated at 27°C to 32°C for

2 weeks.

Second regeneration: by employing the same regeneration medium as that used for the first regeneration, an incubation was performed at 27°C to 32°C for 2 more weeks.

#### 1.6 potting

The transformants which had regenerated were placed on a rooting medium (an MS medium containing no hormones, supplemented with hygromycin (25m g/l). After the growth of roots was confirmed, the transformants were potted (Figure 2).

Example 2: transformation of a rice plant using a conventional method

For comparison with the method described in Example 1, a rice plant transformation according to a conventional method was performed as follows, using Nipponbare as the material to be transformed.

#### 2.1 callus induction

After removing the husks of the seeds of



Nipponbare, the seeds were sterilized and sown on a callus induction medium (an N6D medium containing 2m g/l of 2,4-D), and incubated at 30°C under light. About 4 weeks after the beginning of callus induction, the grown  
5 calluses derived from blastodisks were used for transformation.

## 2.2 transformation

The resultant calluses were infected with  
10 Agrobacterium EHA101 which was transformed with a plant expression vector pIG121Hm as described in Example 1, and incubated on an 2N6-AS medium in the dark at 28°C for 3 days to effect co-culturing.

## 15 2.3 bacterium elimination and selection

By using an N6D medium containing 500m g/l of carbenicillin, the Agrobacterium was washed off the calluses. Next, the selection of the transformed callus was performed under the following conditions.

20

First selection: the calluses were placed on an N6D medium containing 2 mg/l of 2,4-D which was supplemented with carbenicillin (500 mg/l) and hygromycin (50 mg/l), and incubated at 27°C to 32°C for

2 weeks.

Second selection: the calluses were placed on an N6D medium containing 2 to 4 mg/l of 2,4-D which was supplemented with carbenicillin (500 mg/l) and hygromycin (50 mg/l), and incubated at 27°C to 32°C for 2 more weeks.

#### 2.4 regeneration, rooting and potting

The selected transformed seeds were allowed to regeneration under similar conditions to those employed in Example 1, and all the processes down to potting were carried out.

#### 2.5 results

Figure 3 comparatively illustrates a transformation example according to a conventional method, and a transformation example according to the method of the present invention. The number of days which were required after sowing until the potting of the transformants was about 90 days for the conventional technique, as opposed to about 50 days for the method according to the present invention (Figure 3 (a)). A comparison on about the 50th day since sowing indicated

that, while the transformants obtained according to the method of the present invention were ready to be potted, transformants obtained according to the conventional method were still in the process of regeneration  
5 (Figure 3 (b)). In summary, the method according to the present invention reduced the period required for transformation to about two-thirds or less of that required by the conventional method.

#### 10 INDUSTRIAL APPLICABILITY

According to the present invention, an improved Agrobacterium-mediated transformation method for monocotyledons is provided. According to the method of the present invention, intact seeds of a plant which is  
15 intended to be transformed are infected with an Agrobacterium which contains a desired recombinant gene. The use of the present invention enables a more efficient and quicker creation of a transformed plant.

20250-9454T660

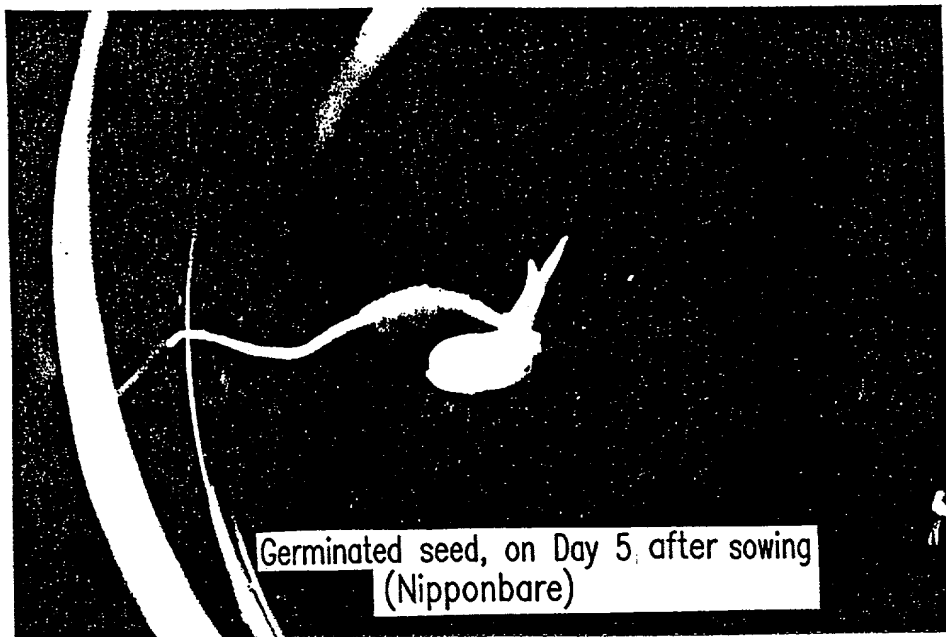
CLAIMS

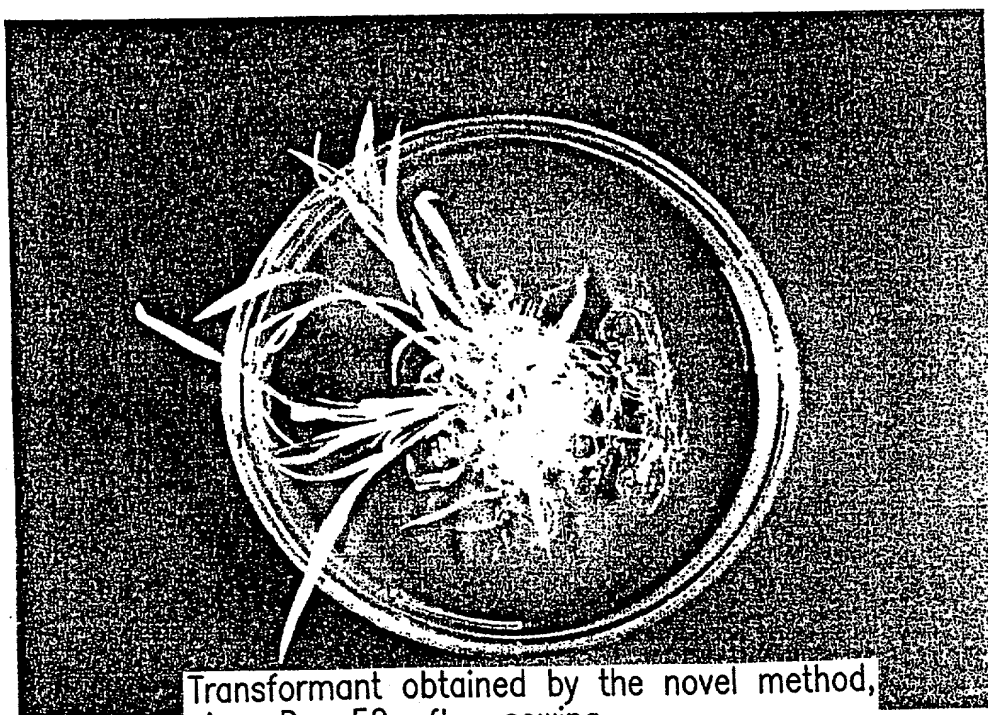
1. A method for transforming a monocotyledon, comprising  
a step of infecting an intact seed with an Agrobacterium  
5 which contains a desired recombinant gene.
2. A method according to claim 1, wherein the seed is a  
seed on the fourth or fifth day after sowing.
- 10 3. A method according to claim 2, wherein the seed is  
a germinated seed.
4. A method according to claim 3, wherein the  
monocotyledon is a plant of the family Gramineae.
- 15 5. A method according to claim 4, wherein the plant of  
the family Gramineae is rice.

ABSTRACT

There is provided an Agrobacterium transformation  
method for monocotyledons, comprising a step of infecting  
5 an intact seed.

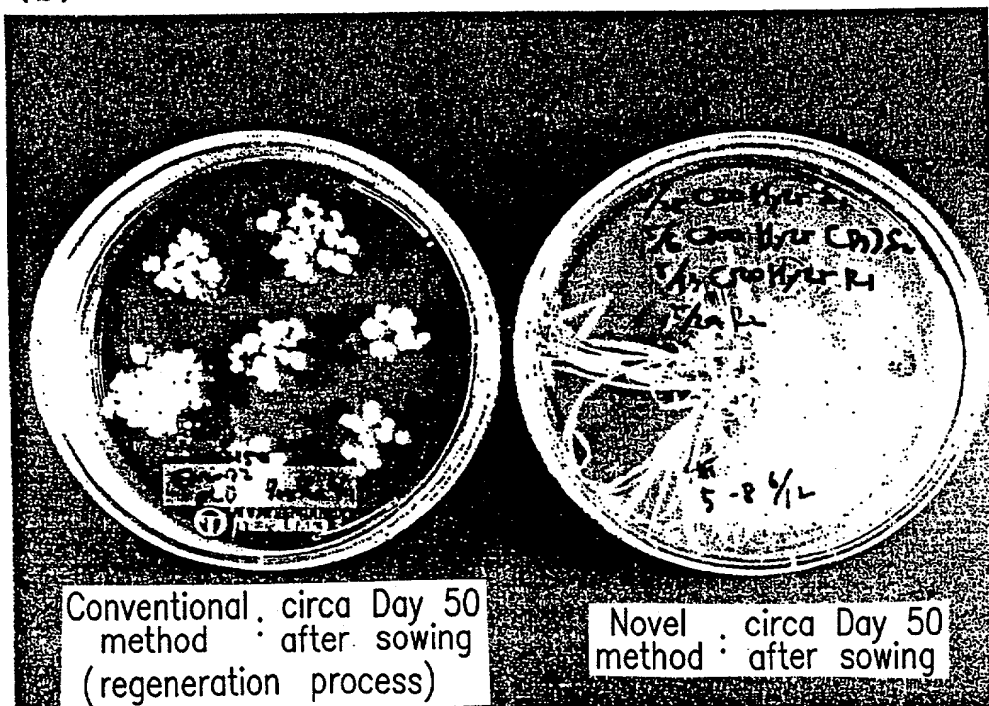
202760-9494660

*FIG. 1*

*FIG. 2*

Transformant obtained by the novel method,  
circa Day 50 after sowing

3/3





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**DECLARATION FOR UTILITY OR  
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PATENT APPLICATION  
(37 CFR 1.63)****UNEXECUTED**☒ Declaration

Submitted

OR

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Submitted after Initial

with Initial

Filing

Filing (surcharge  
(37 CFR 1.16 (e))  
required)

Attorney Docket Number

YAM 2 0010

First Named Inventor

TANAKA, Hiroshi

**COMPLETE IF KNOWN**

Application Number

/

Filing Date

Group Art Unit

Examiner Name

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**ULTRA-FAST TRANSFORMATION TECHNIQUE FOR MONOCOTYLEDONS**

the specification of which

(Title of the Invention)

☐

is attached hereto

OR

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was filed on (MM/DD/YYYY)

07/22/1999

as United States Application Number or PCT International

Application Number

PCT/JP99/03920

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(if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

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# DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)

## UNEXECUTED

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Attorney Docket Number

YAM 2 0010

First Named Inventor

TANAKA, Hiroshi

## COMPLETE IF KNOWN

Application Number

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Filing Date

Group Art Unit

Examiner Name

As a below named inventor, I hereby declare that:

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I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

ULTRA-FAST TRANSFORMATION TECHNIQUE FOR MONOCOTYLEDONS

the specification of which

(Title of the Invention)

☐ is attached hereto

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as United States Application Number or PCT International

Application Number PCT/JP99/03920 and was amended on (MM/DD/YYYY) (if applicable).

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Name of Sole or First Inventor:

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## DECLARATION

ADDITIONAL INVENTOR(S)  
 Supplemental Sheet  
 Page 4 of 5

Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor					
Given Name (first and middle [if any])		Family Name or Surname					
Naoto		SHIBUYA					
Inventor's Signature						Date	
Residence: City	Tsukuba-shi	State	Ibaraki	Country	JAPAN JPX	Citizenship	JAPAN
Post Office Address	c/o National Institute of Agrobiological Resources						
Post Office Address	Ministry of Agriculture, Forestry and Fisheries 2-1-2, Kannondai						
City	Tsukuba-shi	State	Ibaraki	ZIP	305-8602	Country	JAPAN
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor					
Given Name (first and middle [if any])		Family Name or Surname					
Haruko		ONODERA					
Inventor's Signature						Date	
Residence: City	Tsukuba-shi	State	Ibaraki	Country	JAPAN	Citizenship	JAPAN
Post Office Address	c/o National Institute of Agrobiological Resources						
Post Office Address	Ministry of Agriculture, Forestry and Fisheries 2-1-2, Kannondai						
City	Tsukuba-shi	State	Ibaraki	ZIP	305-8602	Country	JAPAN
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor					
Given Name (first and middle [if any])		Family Name or Surname					
Kazuko		ONO					
Inventor's Signature						Date	
Residence: City	Tsukuba-shi	State	Ibaraki	Country	JAPAN	Citizenship	JAPAN
Post Office Address	c/o National Institute of Agrobiological Resources						
Post Office Address	Ministry of Agriculture, Forestry and Fisheries 2-1-2, Kannondai						
City	Tsukuba-shi	State	Ibaraki	ZIP	305-8602	Country	JAPAN

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**DECLARATION****ADDITIONAL INVENTOR(S)**  
**Supplemental Sheet**  
Page 5 of 5

Name of Additional Joint Inventor, if any:

☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])

Family Name or Surname

AkemiTAGIRIInventor's  
Signature

Date

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Name of Additional Joint Inventor, if any:

☐ A petition has been filed for this unsigned inventor

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Family Name or Surname

YaekoNISHIZAWAInventor's  
Signature

Date

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Name of Additional Joint Inventor, if any:

☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])

Family Name or Surname

Inventor's  
Signature

Date

Residence: City

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